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EARLY *IN VITRO* LIPOPOLYSACCHARIDE-INDUCED SERUM PROTEIN AGGREGATION IN TOLERANT SERUM

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

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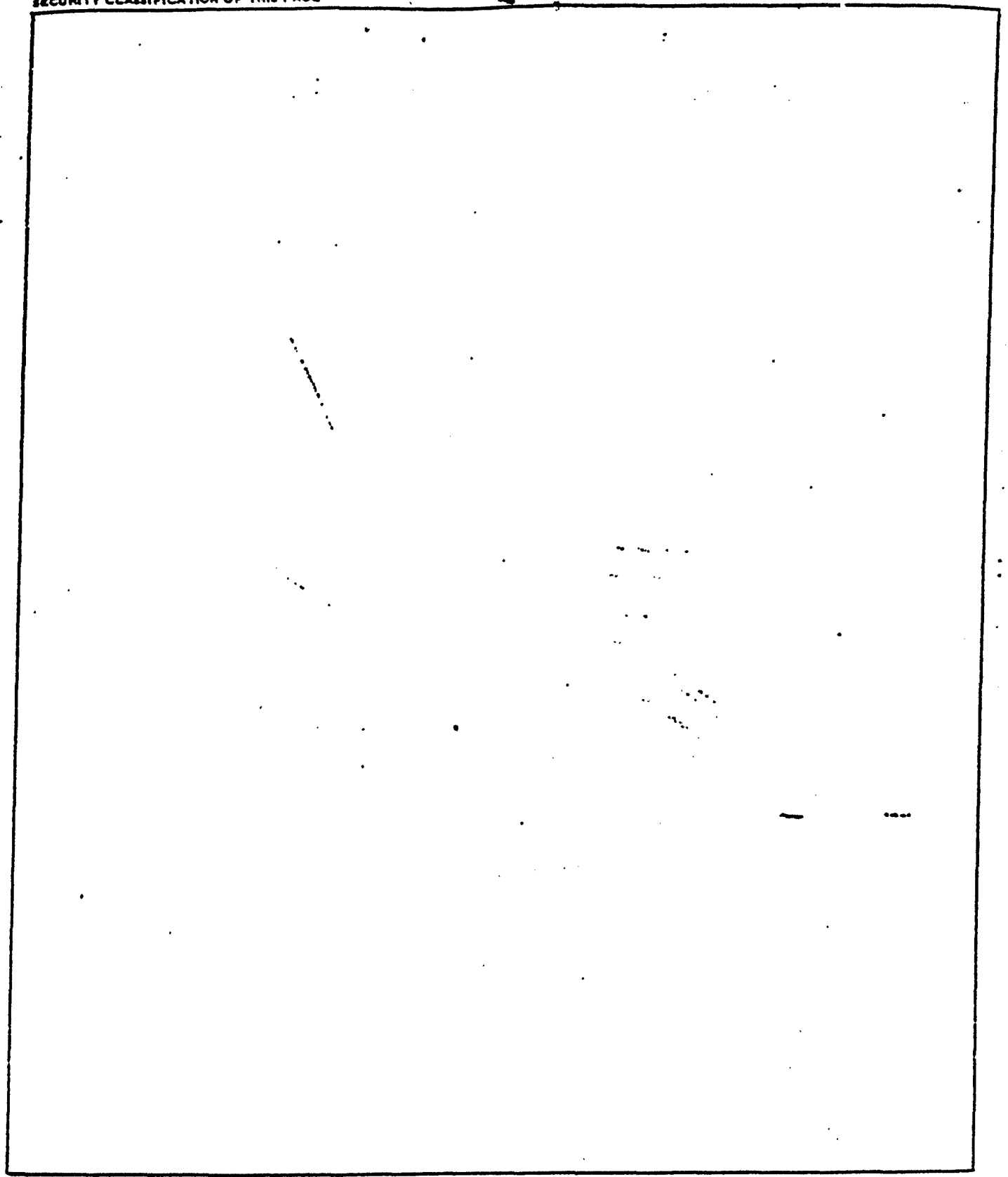

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Early In Vitro Lipopolysaccharide-Induced Serum Protein Aggregation In Tolerant Serum

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Abstract

In a rat LPS shock model, an intravenous injection of LPS (3 - 30 mg/kg body weight) corresponds to an estimated peak load of 0.13 - 1.3 mg LPS/ml serum. We tested the effects of such LPS concentrations *in vitro*. Rat serum was incubated with LPS (0.1 - 2.0 mg LPS/ml serum) at 37°C. The exact amount of precipitate (ppt) depended on both LPS concentration and incubation time. By varying the incubation time (5 sec - 60 min), with a set concentration of LPS (0.5 mg/ml), we found differences between normal and tolerant rat serum. For normal serum, the amount of ppt was the same at all incubation time points. With tolerant serum, the amount of ppt after 5 min incubation was twice the amount from normal serum. Much of this enhanced ppt faded by the 10 min incubation time point. Precipitated protein was freed of LPS by NaCl/EDTA extraction, and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Some protein from normal serum aggregates with LPS, as shown by SDS-PAGE bands at 18, 28, 68, 110, and 190 kDa. Two particular components, at 65 Kda (preceding rat albumin) and 43 kDa, distinguished tolerant from normal serum. These precipitated from tolerant serum within 5 sec of incubation, but required much longer incubation (2 - 3 min) to be precipitated from normal serum. Neither band was detected using non-reducing conditions. Unlike normal serum, tolerant serum interacts with LPS *in vitro* in a dynamic way, which may hold clues to the protective effects of tolerance.

Introduction

Both the structure and function of lipopolysaccharide (LPS), the toxic outer membrane component of gram-negative bacteria, are greatly affected by the methods used to solubilize it. When LPS is suspended in PBS it aggregates to form tubes, sheets, and vesicles (Coughlin, 1983). In a commonly used animal model of LPS shock, LPS is dissolved in PBS and then administered as an intravenous bolus to rats. The fate of the resultant LPS aggregates in the serum and their pathophysiologic consequences are intricately interwoven with their complex interactions with serum components in this animal model. Many different serum components have been shown to bind LPS; thse include serum proteins (Skarnes, 1978; Yajima, 1986, Warren, 1987, Tobias, 1988) lipoprotein micelles (Ulevitch, 1979; Munford, 1981; Munford, 1982; Munford, 1985; Flegel, 1989; Cavaillon, 1990), and various cell types. Each of these components has been postulated to serve different roles in the pathophysiology and clearance of LPS.

Although it is well-known that serum can neutralize LPS serum (Oroszlan, 1966; Warren, 1985), the exact mechanism of neutralization is unknown. Upon challenge with LPS, individual rats routinely show a wide variation in sensitivity. In our experience with a lethal endotoxin shock model, for example, an iv dose of 12.5 mg LPS/kg caused anywhere from 40 to 90% lethality (Figure 1). We speculate that the variability in lethality was due in part to different levels of serum neutralization among the rats.

In additon to the normal neutralization of LPS toxicity by serum proteins, serum compounds have been previously implicated in the process of LPS tolerance. Tolerance is the profound protective response that follows exposure to small quantities of LPS (Urbaschek, 1984; Old, 1985). As compared with normal serum, tolerant serum has an enhanced ability to detoxify LPS (Yamaguchi, 1987; Yamaguchi, 1986; Olafsson, 1986; Moreau, 1973). Tolerance occurs within 10 hrs of an initial sub-lethal tolerizing dose, and can persist for the following three to four days (Creech, 1948; Freudenberg, 1988). We have found that rats made tolerant to LPS can survive a dose of LPS that is two to three times greater than one that would kill a normal rat.

Different mechanisms have been advanced to exlain tolerance. White cells, such as macrophages and leukocytes, can become desensitized to the stimulating action of LPS. Tolerizing doses of LPS may enhance clearance by causing the release of certain cytokines which can augment the response of the reticuloendothelial system. The intrinsic ability of the serum to neutralize LPS can be enhanced after the induction of tolerance, either through stimulation of serum proteins already present, or through the induction of new serum proteins. One potential mechanism to explain serum protein-induced tolerance may be found in work of Tobias and Ulevitch.

Tobias and Ulevitch showed that as LPS is incubated in serum from normal rabbits (0.01 - 1 mg LPS/ml serum), the LPS micelles react with the serum components by a two-step process: serum-dependent dissolution of LPS

micelles followed by protein or lipoprotein binding (Rudbach, 1966; Ulevitch, 1978). In the serum from tolerant rabbits, the rate of binding to the lipoproteins is significantly slowed by the formation of an intermediate LPS-protein complex. The particular protein responsible for this delay in lipoprotein uptake, since named the endotoxin binding protein, is unique to the serum of tolerant animals.

In the rat model of LPS shock, a large quantity of LPS is injected into the bloodstream to provoke shock. For our *in vitro* assay, we estimate that the peak serum concentration of a bolus iv injection, 3 - 30 mg/kg, corresponds to a serum concentration of 0.1 - 2.0 mg LPS/ml serum. At these concentrations, serum proteins co-aggregate with the LPS micelles. Unlike LPS neutralization by serum or lipoprotein uptake of LPS, this phenomenon of LPS-induced serum protein aggregation has not been closely studied. Doing so may help us to understand what happens in the first moments after LPS has been injected into the serum. For example, the formation of LPS-protein aggregates could lead to perfusion abnormalities at the capillary level or macrophage activation after phagocytosis of the LPS. Alternatively, certain serum proteins may complex with LPS to form large protein-LPS aggregates in which LPS has been neutralized.

By using the serum concentrations that are associated with bolus LPS administration, we also may be able to understand the difference between the acute endotoxin model and Gram-negative shock. This may help us to understand the physiology of acute LPS shock, especially the phenomena of

hypoperfusion and diffuse intervascular coagulation. Since normal rats are much more resistent to the toxic effects of LPS than either normal rabbits or humans, we hope to determine if this difference is based in the serum. Finally, by comparing serum from normal and tolerant rats, we hope that our study will reveal a unique response of tolerant serum to incubation with LPS.

Materials and Methods

Preparation of Normal and Tolerant Serum

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY), 280 to 330 g each, were placed in isolettes upon their arrival from the vendor. They were maintained in a light and temperature controlled environment, and were watered and fed standard rat chow *ad libitum*. LPS (*E. coli* 0111:B4, Sigma Chemical Company, St. Louis, MO), made up freshly for each experiment, was suspended by gentle rocking for 24 hr in 0.15 *M* PBS (0.15 *M* NaCl, 0.020 *M* sodium phosphate, pH 7.4). The injection mixture was briefly vortexed just before injection. Rats were made tolerant by intraperitoneal injection each day, for three days, with 0.9 mg/kg LPS. Unchallenged rats received only PBS. On the fifth day, serum was obtained. Rabbit serum, both normal and tolerant, was a gift of Dr. H. Shaw Warren (Warren, 1986).

To collect serum, the rats were anesthetized with "Equithesin" (50 ml Nembutal, 87 ml Propylene glycol, 23.8 ml ethanol, 10.6 g Chloral hydrate, 5.2 Magnesium sulfate; with added saline to bring the total volume up to 250 ml), and then exsanguinated *via* an abdominal aortic puncture. Blood was collected in a Vacutainer, and then gently rocked for 30 minutes. Samples were spun at 4000 rpm for 30 min, and serum withdrawn. All serum samples were frozen in aliquots at -70°C until used.

LPS Shock Model

Under anesthesia by halothane inhalation (2-Bromo-2-chloro-1,1,1-trifluoroethane, Halocarbon Laboratories, North Augusta, SC), rats were injected intravenously (*iv*) via the dorsal vein of the penis (in a fluid load of 3.0 ml/kg). The *in vivo* challenge to the rats ranged from 3.0 to 30 mg/kg of the LPS. Halothane was then removed, and the animals quickly awoke. They were maintained on water and standard rat chow. Survival was determined at 48 hrs.

LPS-serum protein aggregation assay

Estimated *in vivo* peak serum LPS levels, following a bolus administration of LPS were matched to *in vitro* concentrations. Based on the average rat blood volume of 50 ml/kg, with a hematocrit of 45%, an injection of 22.5 mg LPS/ml PBS (lethal dose 80%) gave an estimated peak serum concentration of 1 mg LPS/ml serum. In our *in vitro* experiments, serum was adjusted with phosphate buffered saline, pH 7.4, containing no calcium or magnesium, (PBS), to a standard final protein concentration of 17.5 mg/ml. (Such cations can change the physical state of LPS, and encourage serum protein precipitation.) LPS, was dissolved in PBS by overnight gentle agitation, and then added to serum, 1 : 1 (v/v). The mixture was incubated at the appropriate temperature (0, 25, 37°C). After centrifugation (Beckman Microfuge, 0°C, 30 - 40 min), a precipitate, the

"primary pellet," was obtained. The tips of the Eppendorf tubes containing the pellets were immediately cut off and carefully washed twice in PBS (0°C). This was followed by gentle blotting with Kimwipes to remove excess fluid.

In some experiments, serum was incubated with EDTA (20 m*M*) before the addition of the LPS. In other experiments, the serum was heat-treated (56°C, 45 min) or incubated with lead acetate (2 mg/ml) before being exposed to LPS. Lead acetate has been used traditionally to sensitize rats to endotoxin challenge (Yamaguchi, 1987). Different types of serum were used in these experiments: rat (normal and tolerant) and rabbit (normal and tolerant). In another set of experiments with a fixed concentration of LPS (0.5 mg/ml), the time of incubation was varied from 5 sec to 60 min.

Protein concentration in the primary pellets was determined by the Folin reagent, using bovine serum albumin (BSA) as the standard (Lowry, 1951). The sample was solubilized by addition of sodium hydroxide (0.05 M) and pulsed sonication (pulse time 900 msec, for a total time of 90 sec), followed by heating (90°C) and vortexing.

In prepare the samples for gel electrophoresis, it was necessary to remove the LPS from the protein in the LPS-induced aggregate. The primary precipitate was suspended in 800 μL of 1 M NaCl and 10 m*M* EDTA in PBS. This was followed by brief vortexing and water-bath incubation (37°C, 60 min) with agitation. Then, the sample was precipitated with 400 μL of ice-cold 80% TCA. This mixture was vortexed, chilled (0°C, 30 min), and then centrifuged (Beckman

Microfuge, 0°C, 45 min). A small white pellet was obtained, and the supernatant discarded. A 2 min centrifugation with a Brinkmann Tabletop centrifuge was done at room temperature, and any remaining fluid was gently removed by blotting.

To run SDS-PAGE mini-gels, sample buffer was added (with or without reducing agent.) The pellet was suspended by multiple drawing and pipeting, followed by vortexing (30 s). To dissolve the pellet completely, samples were refrigerated overnight (0 - 4°C) with gentle agitation. Just before running the gel, samples were heated (60°C, 20 min) and then spun down (Beckmann Tabletop, room temperature, 1 min). The samples were electrophoresed in the presence of SDS using 4 - 20% gradient or 7.5% gels until 5 min after the dye front ran off.

Results

LPS-shock lethality: Normal and tolerant rats

The lethal threshold for LPS injection was about ~ mg/kg, and at LPS doses above 25 mg/kg, lethality approached 100% [Figure 1]. The toxic effects of LPS injection, as used in this study, were consistent with previous reports (Mathison, 1979, Wichterman, 1980). The time until death ranged from 3 to 48 hr, with most (over 80%) dying 3 to 6 hr after injection. Median survival time for the LPS injected group was 4 hr. After rats were made tolerant, they could survive an LPS challenge that was two to three times greater than that necessary to cause the same lethality in normal rats. For instance, when rats were challenged with 50 mg/kg iv LPS, (2 times the LD_{100} dose), all tolerant rats survived (5/5), as compared with one survivor (1/5) in a normal group that had received daily ip injections of PBS in lieu of LPS. This difference was statistically significant ($p < 0.05$, Chi-square).

LPS-serum protein aggregation assay

For normal rat serum, the amount of precipitate depends on the LPS concentration [Figure 2]. Following 1 hr incubation, the precipitation curve at 1 - 2 mg LPS/ml serum represents about 0.5 % of the total serum proteins. Within

the range of 0.1 to 2 mg LPS/ml, the precipitation curve is not very flat, taking on the appearance of the buffer region of a standard acid-base curve. Beyond 2 mg LPS/ml serum, the amount of precipitate greatly increases [Figure 2].

There are some manipulations of rat serum that can greatly dampen its precipitating ability, including incubation with 20 m*M* EDTA [Table 1], heat pretreatment (56°C, 45 min), [Table 1], and incubation on ice [data not shown]. In our *in vitro* system, lead acetate (2.0 mg/ml) has no effect on the precipitation phenomenon [data not shown].

Normal rat and rabbit precipitate similar amounts of protein when incubated with LPS at 37°C for 60 min [Figure 5]. Serum from a tolerant rat or rabbit, when incubated with LPS, formed more precipitate than that from the corresponding normal animal. This was most pronounced at LPS concentrations above 0.5 mg/ml.

As we varied the time of incubation, using a 0.5 mg/ml LPS concentration, differences in protein precipitation between normal and tolerant serum appeared [Figure 6]. For the rat serum, the difference is pronounced at 5 minutes. In preliminary results using rabbit serum, the difference between normal and tolerant were much less pronounced [Figure 7].

We also ran SDS-PAGE on the precipitates. LPS induces shifts and distortions in the bands during electrophoresis. For example, when BSA was electrophoresed in the presence of LPS, the band ran more slowly than controls, and had a jagged appearance. Reproducibility of band position was

also a problem with the "primary pellets." All these electrophoretic anomalies were directly proportional to the amount of pellet protein loaded. However, once the LPS was removed from the "primary pellet" with 1 M NaCl, 10 m*M* EDTA, and TCA reprecipitation these electrophoretic problems vanished.

Although there are many bands observed in the precipitate (major bands at 18, 28, 43, 65, 68, 110, and 190 kDa), time of incubation affected the electrophoretic pattern of two bands in particular [Figure 8]. After only a brief incubation (5 sec), the tolerant serum ppt had prominent components at 65 kDa (preceding rat albumin) and 43 kDa. These bands eventually could be seen in the normal serum ppt, but only after 2 - 3 min incubation. Neither of these bands was present on a non-reducing SDS-PAGE gel of the ppt, suggesting that they are components of a much higher molecular weight species.

Discussion

When LPS is solubilized in PBS and administered as an iv bolus, a dose of 3 mg/kg is the beginning of the lethal range. In our *in vitro* aggregation assay, formation of a protein precipitate becomes noteworthy at an LPS concentration of 0.1 mg LPS/ml serum, which is equivalent to an LPS challenge of 2.2 mg/kg. Under these conditions potentially toxic aggregates of LPS may remain within the circulation (Galanos and Lüderitz, 1976).

These circulating LPS aggregates have complex interactions with various serum components, and consequently may result in multiple outcomes. On the one hand, the immediate toxicity of LPS might be checked by protein and lipoprotein binding (Olafsson, 1986; Yamaguchi, 1986; Yamaguchi, 1987). On the other hand, LPS-protein microemboli, similar to the aggregates we observed *in vitro*, could be trapped in constricted microvessels where they may cause local stasis and poor perfusion. Such tiny emboli may remain as encapsulated toxin, just waiting to be released upon macrophage phagocytosis (Yamaguchi, 1982). Macrophages, including those residing within the reticuloendothelial system might be activated, contributing to long-term inflammatory damage (Yamaguchi, 1987; Regel, 1987). Furthermore, if LPS that is bound up with serum proteins or lipoproteins remains within the body longer than "free" LPS, protracted toxicity could result. Munford *et al* (Munford, 1985) have shown that LPS-high density lipoprotein (HDL) complexes have a prolonged half-life in the

serum, do not bind to blood cells, and are taken up slowly by tissues that use the HDL-cholesterol for purposes such as synthesis of steroid hormones.

The present study was designed to understand what happens to the LPS micelles when they are introduced into the serum at this lethal concentration. We have studied the co-aggregation of serum proteins and LPS micelles, monitored the binding of serum proteins, the conditions that effect serum protein co-aggregation, and have begun to identify the co-aggregating proteins.

We have shown that LPS *in vitro* can induce serum-protein aggregates. The LPS-serum proteins aggregate is substantially diminished by heat pre-treatment (56°C, 45 min). In a similar way, the LPS neutralizing ability of serum can be lost by pre-heating the serum (Yamaguchi, 1986). EDTA has been shown to augment the neutralizing capacity of serum (Skarnes, 1966) and the binding of LPS to HDL (Ulevitch, 1978). EDTA inhibits the formation of ppt in our assay, suggesting that neutralization may not be associated with serum protein-LPS aggregation or binding to HDL. Ulevitch showed that LPS, in the presence of 20 m*M* EDTA, can overwhelm the capacity of serum HDLs to bind LPS [Figure 9] at LPS concentrations greater than 0.8 mg/ml. This further suggests that our ppt is not due to LPS-HDL binding, but is spillover from the HDLs. Finally, the serum aggregation curve in Figure 2 has the shape of a standard acid-base buffer curve. Beyond an LPS concentration of 2.0 mg/ml, which is equivalent to a fully lethal dose in the rat model, the amount of ppt that forms with increasing LPS concentration rises substantially. It appears that once serum protein

aggregation with LPS becomes predominant, and serum can no longer solubilize the LPS micelles, death is assured in this acute LPS shock model.

Although the HDLs are the final reservoir for LPS in serum (Ulevitch, 1978), they may not play a major role in neutralization. It is noteworthy that the serum lipoproteins, although they readily bind LPS, have a meager *in vitro* neutralizing capability, which is limited to from 0.01 to 2.0 μg/ml (Flegel, 1989; Baumberger, 1991). Others have shown that the clearance of LPS by the reticuloendothelial system, specifically the liver and spleen, is not dependent on HDLs as an LPS carrier (König, 1988). Even if there is some degree of neutralization, binding of the HDLs to LPS is not necessarily beneficial. HDL bound LPS is delivered to the organs that use cholesterol, especially the adrenal glands (Mathison, 1978; Munford, 1981), which are important in modulating the physiological response to shock.

Compared with the serum of normal rats, there is a greater amount of LPS aggregation in tolerant rat serum. In our *in vitro* assay, the amount of protein ppt in tolerant rat serum was more pronounced at all time points. This difference was particularly enhanced after 2 to 10 min of incubation, where tolerant serum produced twice as much precipitate as normal serum at the 5 min incubation point. Since tolerant rat serum causes more ppt than normal rat serum, the serum must either 1.) be less able to solubilize LPS, 2.) have a greater concentration of LPS binding proteins, 3.) have fewer HDLs, or 4.) be hampered in the ability of the HDLs to bind up LPS. In any case, as long as

certain serum proteins surround the LPS micelles, and form a large aggregate, they may be able to mask the toxicity of LPS thereby making the *in vivo* challenge initially less harsh.

What is perhaps most remarkable about the aggregation behavior of tolerant rat serum is the rapid disaggregation that follows the initial heightened aggregation. Tolerant serum, it appears, causes LPS to both rapidly aggregate and then disaggregate, all within the initial 10 minutes of the encounter. It has been shown (Munford, 1981; Warren, 1986) that LPS disaggregation by lipoprotein-free plasma enhances the binding of LPS to HDL.

The dynamic nature of the *in vitro* aggregation in tolerant serum is also seen upon examination of the gel electrophoresis of precipitates. From either normal or tolerant serum, many bands appear in the ppt. In tolerant rat serum, two bands--at 43 and 65 kDa--present themselves within seconds of LPS incubation. For normal rat serum, in contrast, it is only after at least 2 to 3 min of incubation that these same bands also appear. By 30 min incubation, both normal and tolerant serum produce nearly the same amount of precipitate, with a similar pattern of SDS-PAGE bands. For rabbit serum, by contrast, there was neither enhanced ppt in the aggregation assay nor the presence of these two bands upon gel electrophoresis. So, when compared with rabbit serum, rat serum has unique properties.

Our results with precipitation of proteins from either normal or tolerant rat serum seem to mirror to some extent the experiments of Ulevitch (Ulevitch,

1978), who followed the transfer of LPS to serum HDLs in rabbit serum. (One important difference between his experiments and ours, however, is that he routinely included 20 mM EDTA to his serum.) With normal rabbit serum, Ulevitch found that transfer to the HDL fraction occurred quickly, with a half-life of 7 min. In acute-phase or tolerant rabbit serum, by contrast, transfer to the HDLs was delayed by the formation of an intermediate, higher density protein-LPS complex of apparent molecular mass, 950 kDa. For tolerant serum, the half-life of HDL uptake was almost an hour. The 950 kDa complex, which formed after incubation at an LPS concentration of 0.01 mg/ml, contained major reduced protein bands at 50, 61, 64, and 91 kDa. In our ppt, collected at an LPS concentration of 0.5 mg/ml, the major reduced bands are at 18, 28, 43, 65, 68, 110, and 190 kDa. We suspect that enhanced tolerant rat serum precipitation at 5 minutes represents an LPS-protein complex intermediate comparable with the one Ulevitch observed.

As indicated by the comparison between the reducing and non-reducing gels, the 65 and 43 kDa bands are components of a larger serum protein, which may be responsible for delaying the transfer of LPS to the HDLs. This larger serum protein could be α-2-macroglobulin, a tetramer of molecular mass 725 kDa (Yoshioka, 1970; Barnett, 1981; Lonberg-Holm, 1986). Defensins, a class of anti-microbial, LPS-binding proteins manufactured by leukocytes and certain cells in the gut, are transported by α-2-macroglobulins (Panyutich, 1991). During the induction of tolerance, we speculate, defensins are created, bound to

the macroglobulin, and then transported through the serum. If macroglobulins in the serum of tolerant rats are binding to LPS, they could prevent the receptor mediated uptake of LPS, especially by the leukocytes (Regel, 1987), macrophages (Gans, 1976; Freudenberg, 1988; Couturier, 1991), and hepatocytes (Fox, 1990), and so limit LPS's toxicity. Among serum proteins that bind to LPS and therefore are also candidates for the source of the the 43 and 65 KDa bands are: the Ra-reactive factor (Ihara, 1981; Ihara, 1982; Ihara, 1991), the KDO region binding factor (Brade, 1985), antibodies (Skarnes, 1978; Barclay, 1987; Warren, 1987; Warren, 1991), complement factors from both the classical and alternative pathways (Loos, 1990), vitamin D binding protein [also called Gc-globulin or group-specific protein] (Berger, 1990), transferrin (Berger, 1988), albumin (Tesh, 1988), α_2-macroglobulin (Berer, 1990), lysozyme (Ohno, 1991), and the LPS binding protein (Schumann, 1990).

We will continue to study the phenomenon of LPS-induced serum protein aggregation. For example, after pre-heating the serum (56°C, 45 min), we predict that the 65 and 43 kDa bands will be no longer present. Incubation of LPS in tolerant serum at 0°C for either 5 sec or 3 min should also prevent the appearance of the 65 and 43 kDa bands. To understand the role of serum in the disaggregation of the 5 min peak seen in tolerant rat serum, we will collect the "primary pellet" (from 5 min incubation) and incubate it in either normal or tolerant serum. We hypothesize that tolerant serum will have a better ability than normal serum to solubilize the "primary pellet." Even though the literature

plays down any role for antibodies in tolerance, we are concerned that IgM may be responsible for our aggregation/precipitation phenomenon, so we plan to determine the IgM levels of our tolerant serum, in comparison with normal serum. We will check also the LPS levels of these serum samples. Peptide sequence micro-analysis will be performed on rat serum samples obtained by incubation with LPS (0.5 mg/ml) for 5 minutes at 37°C.

In conclusion, rapid serum protein aggregation occurs to a great extent in serum from tolerant rats, as compared with normal serum. For tolerant rats, this enhanced rapid serum protein aggregation with LPS, followed by equally rapid disaggregation over the first 10 min of *in vitro* LPS exposure, might contribute to the substantially improved survival noted in tolerant rats challenged with an otherwise lethal dose of LPS. Tolerant serum, by acting as a buffer against LPS, may be able to neutralize it immediately during the critical period of initial toxin exposure. The difference in the initial aggregation and disaggregation responses of normal and tolerant rat sera may play a role in the enhanced ability of tolerant rats to survive lethal LPS challenge.

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The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, DHEW, Pub. No. (NIH)78-23.

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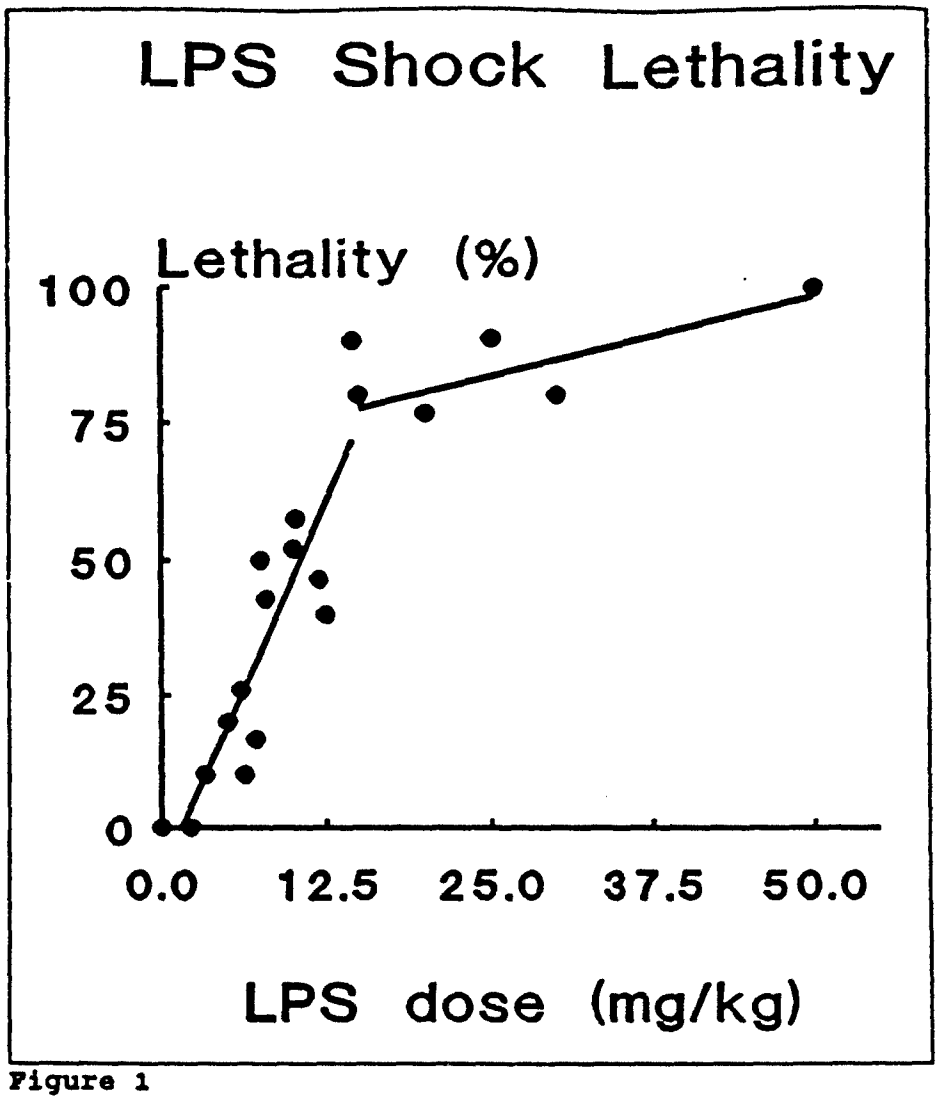

Figure 1

Under anesthesia by halothane inhalation, rats were injected intravenously (iv) via the dorsal vein of the penis with LPS (in a fluid load of 3.0 ml/kg). The in vivo challenge to the rats ranged from 3.0 to 30 mg/kg of the LPS. Halothane was then removed, and the animals quickly awoke. Survival was determined at 48 hrs. Each point represents 8 to 20 rats.

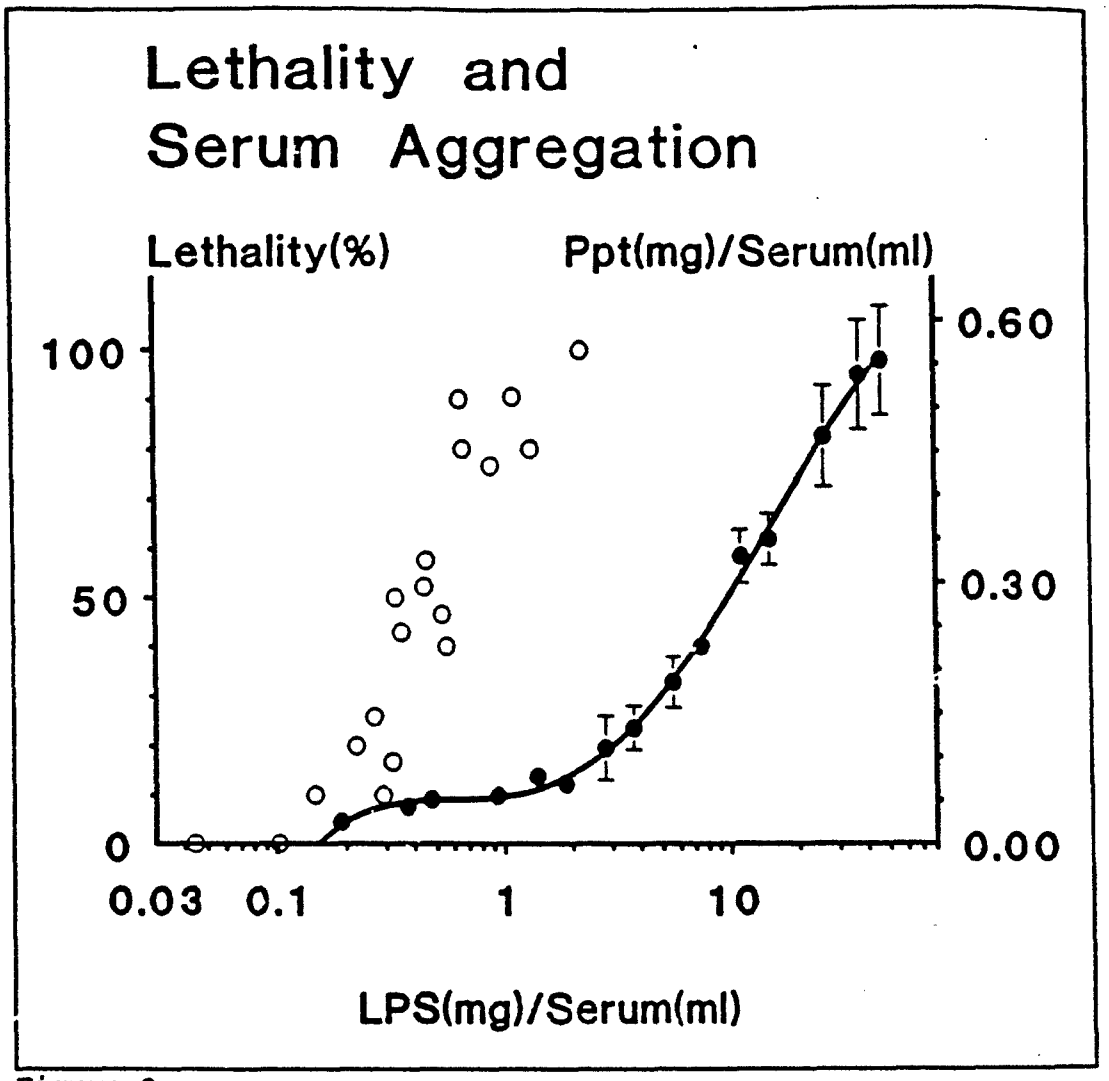

Figure 2

Estimated <u>in vivo</u> peak serum LPS levels, following a bolus administration of LP,S were matched to <u>in vitro</u> concentrations. Based on the average rat blood volume of 50 ml/kg, with a hematocrit of 55%, a lethal injection of LPS (20 to 30 mg/kg), for example, gave an estimated peak serum concentration of about 1 mg LPS/ml serum. Open circles represent 8 to 20 rats for each LPS dose. In our experiments, serum was adjusted to a standard final protein concentration of 17.5 mg/ml with PBS. LPS, was dissolved in PBS by overnight agitation, and then added to serum, 1 : 1 (v/v) and the mixture incubated at the appropriate temperature. After centrifugation (Beckman Microfuge, 0°C, 30 - 40 min), a precipitate, the "primary pellet," was obtained. Protein concentrations were measured using the method of Lowry. Closed circles represent the mean ± standard error of multiple experiments, from 3 to 7 for each LPS concentration.

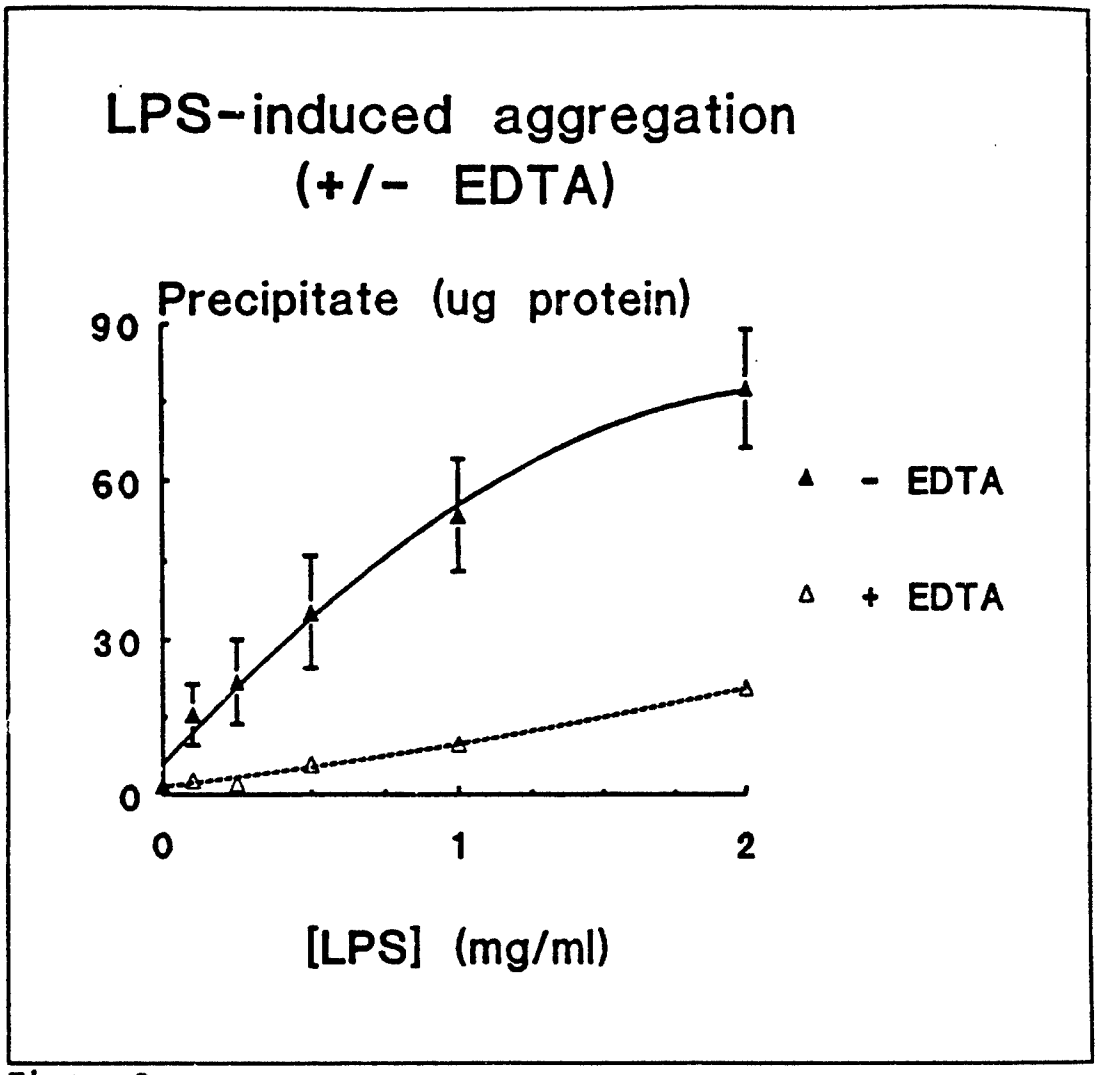

Figure 3

In some experiments, normal serum was incubated with EDTA (20 mM) before the addition of LPS. Each experiment was done in triplicate, and the results are expressed as mean ± standard error.

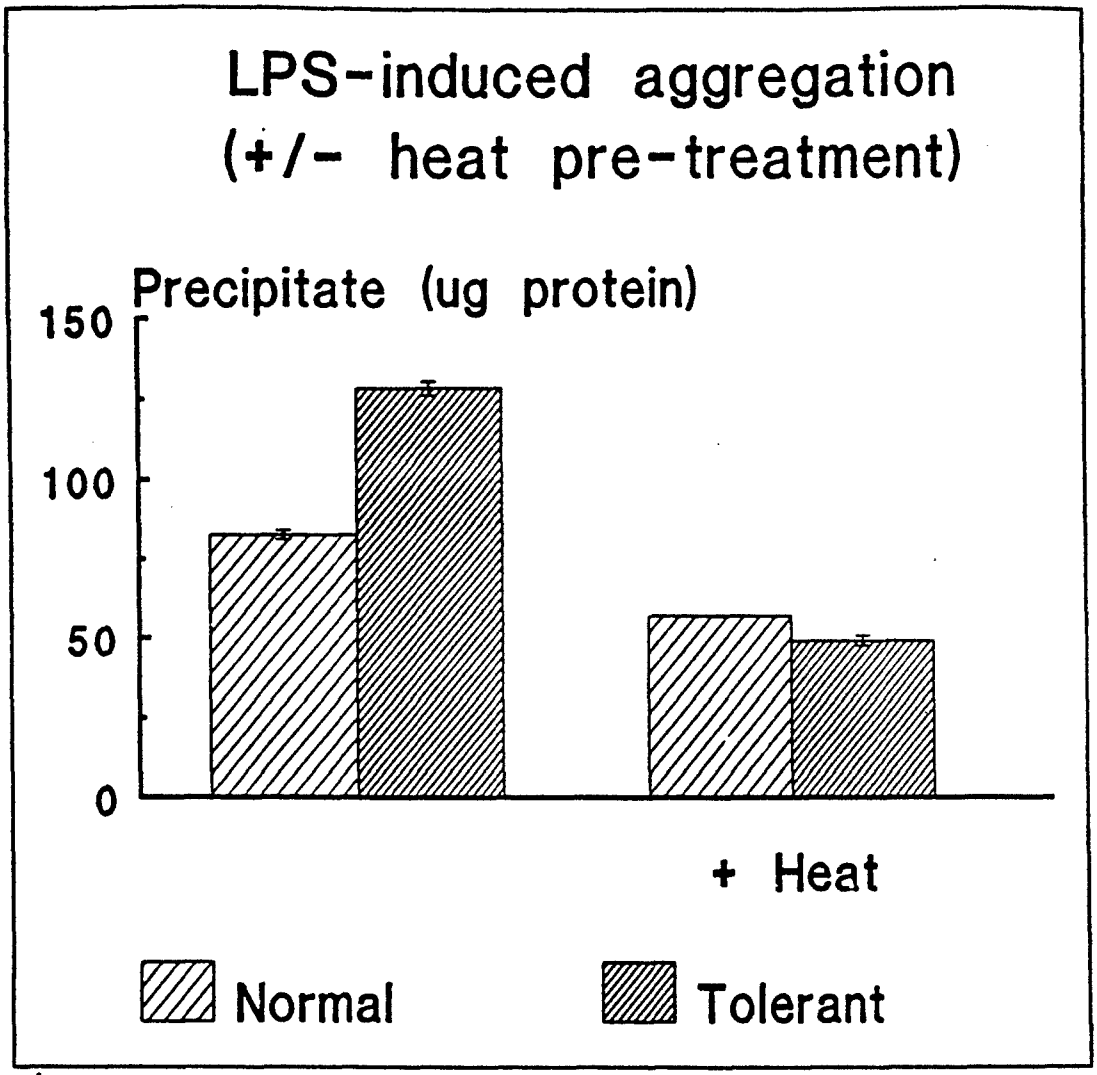

Figure 4

The serum, normal or tolerant, was heat-treated (56°C, 45 min) before being exposed to LPS. Each experiment was done in triplicate, and the results are expressed in mean ± standard error.

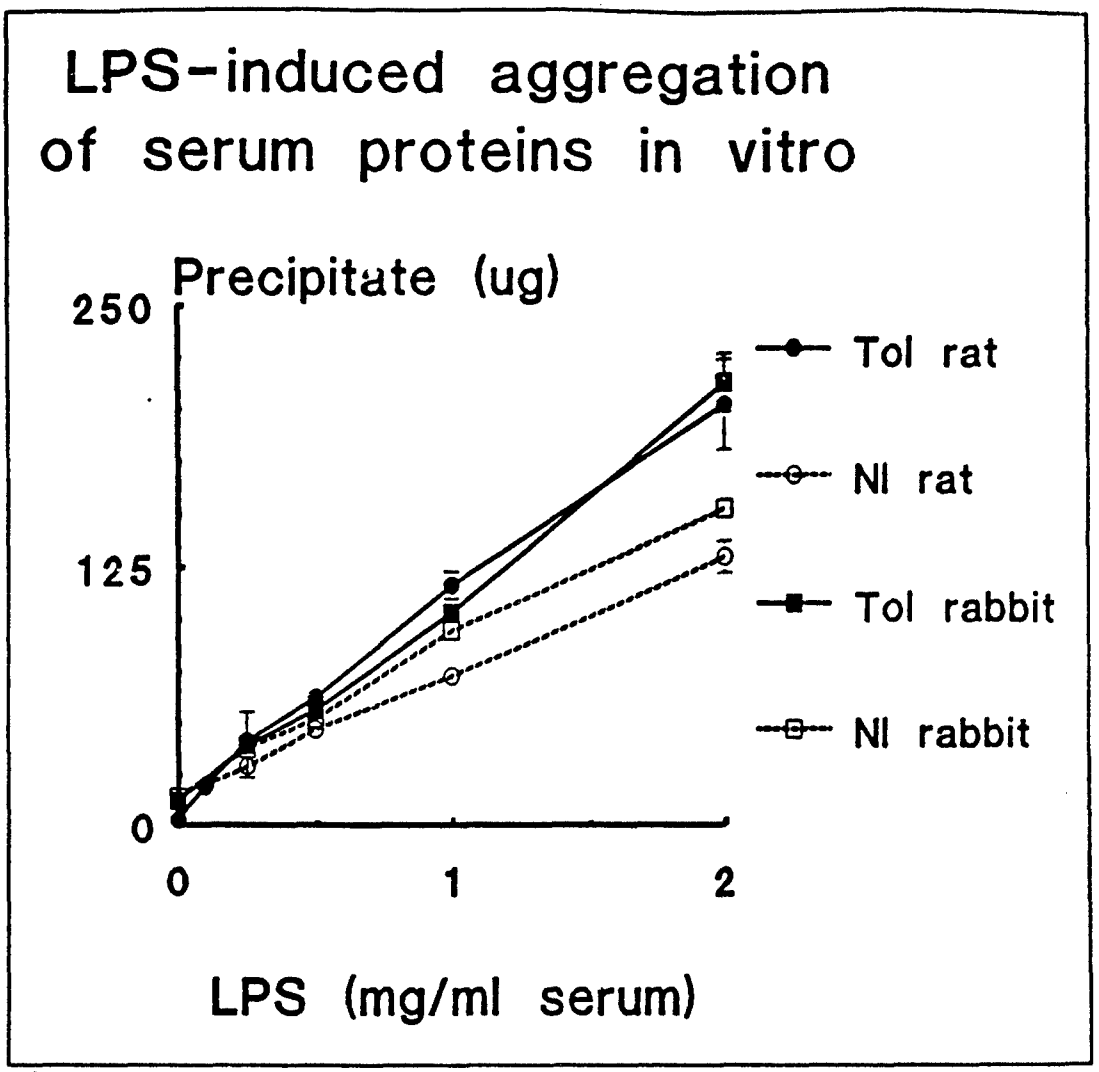

Figure 5

Normal rat (open circle) and normal rabbit (open square) serum precipitate similar amounts of protein when incubated with LPS at 37°C for 60 min. Serum from a tolerant rat (closed circle) or tolerant rabbit (closed square), when incubated with LPS, formed more precipitate than that from the corresponding normal amimal. This was most pronounced at LPS concentrations above 0.5 mg/ml. Each point represents experiments done in triplicate and reported as the mean ± standard error.

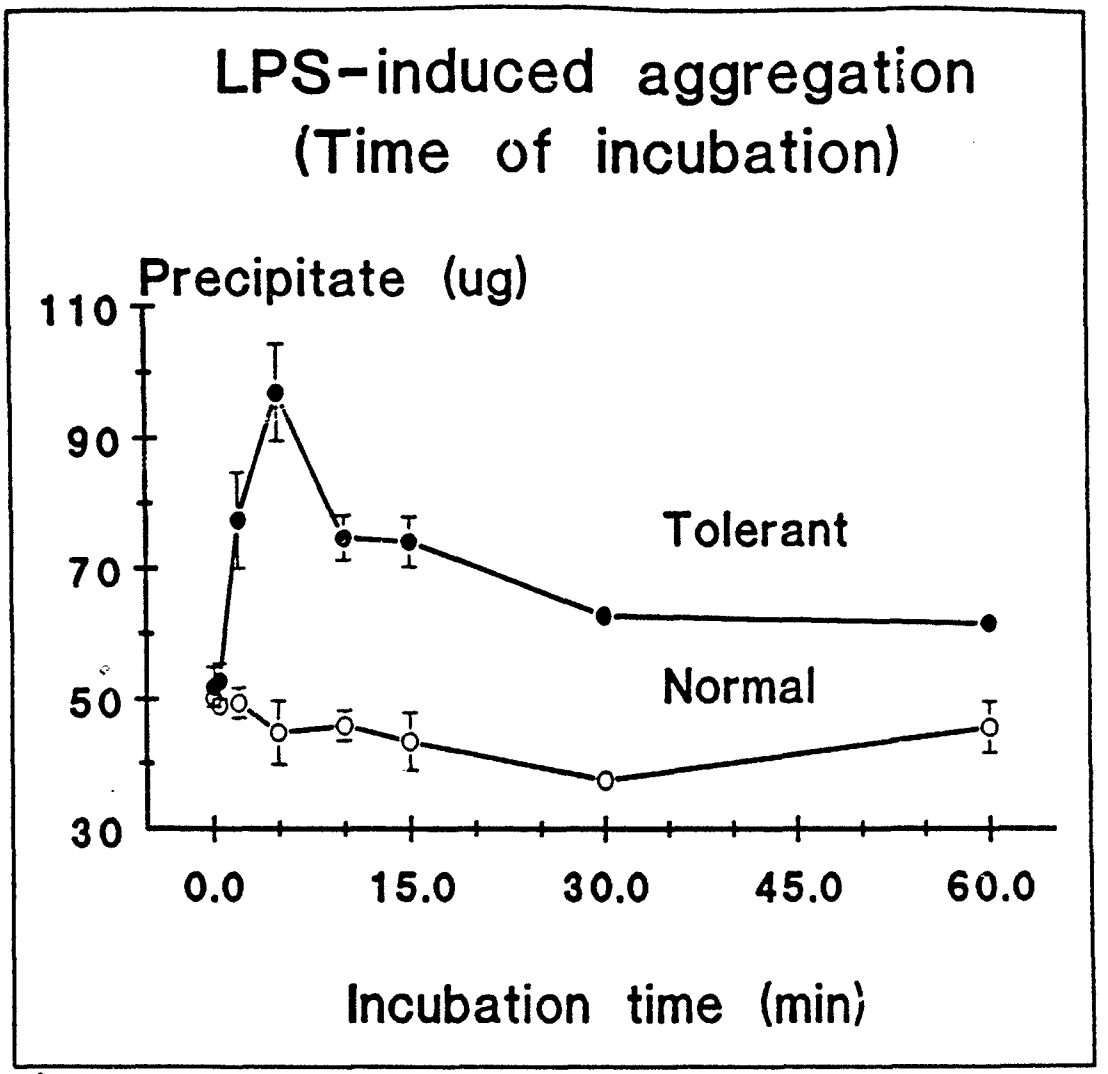

Figure 6

LPS (0.5 mg/ml) was incubated in normal rat (open circle) or tolerant rat (closed circle) serum at 37°C for times ranging from 5 s tc 60 min. The difference in amount of protein precipitation between normal and tolerant serum is most pronounced at 5 min. Each experiment was done in quadriplicate, and the data points represent mean ± standard error.

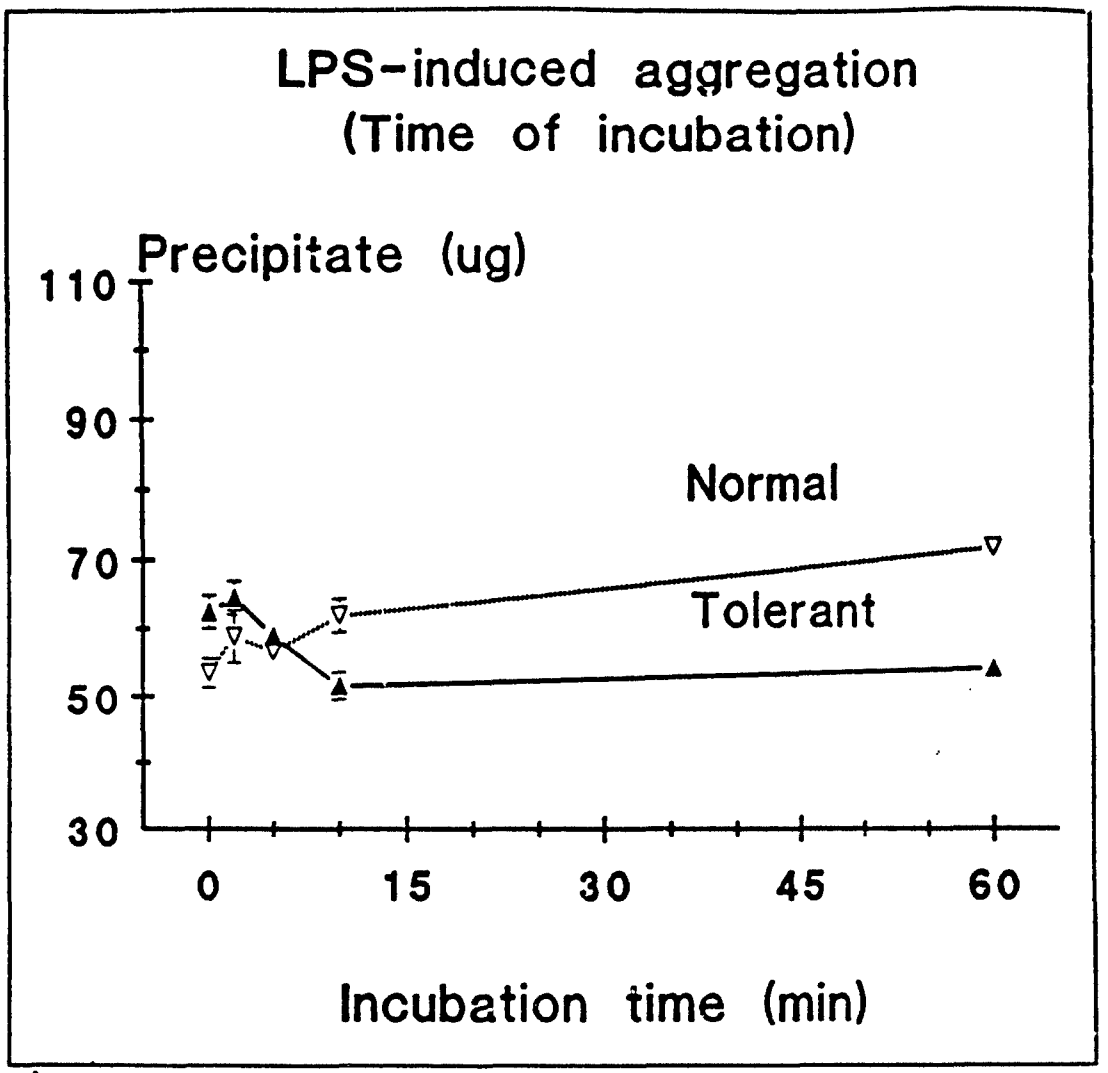

Figure 7

LPS (0.5 mg/ml) was incubated in normal rabbit (open triangle) or tolerant rabbit (closed triangle) serum at 37°C for times ranging from 5 s to 60 min. In preliminary results (done in duplicate) with rabbit serum, the difference between normal and tolerant were much less pronounced than they were in rat serum. The data points represent mean ± standard error.

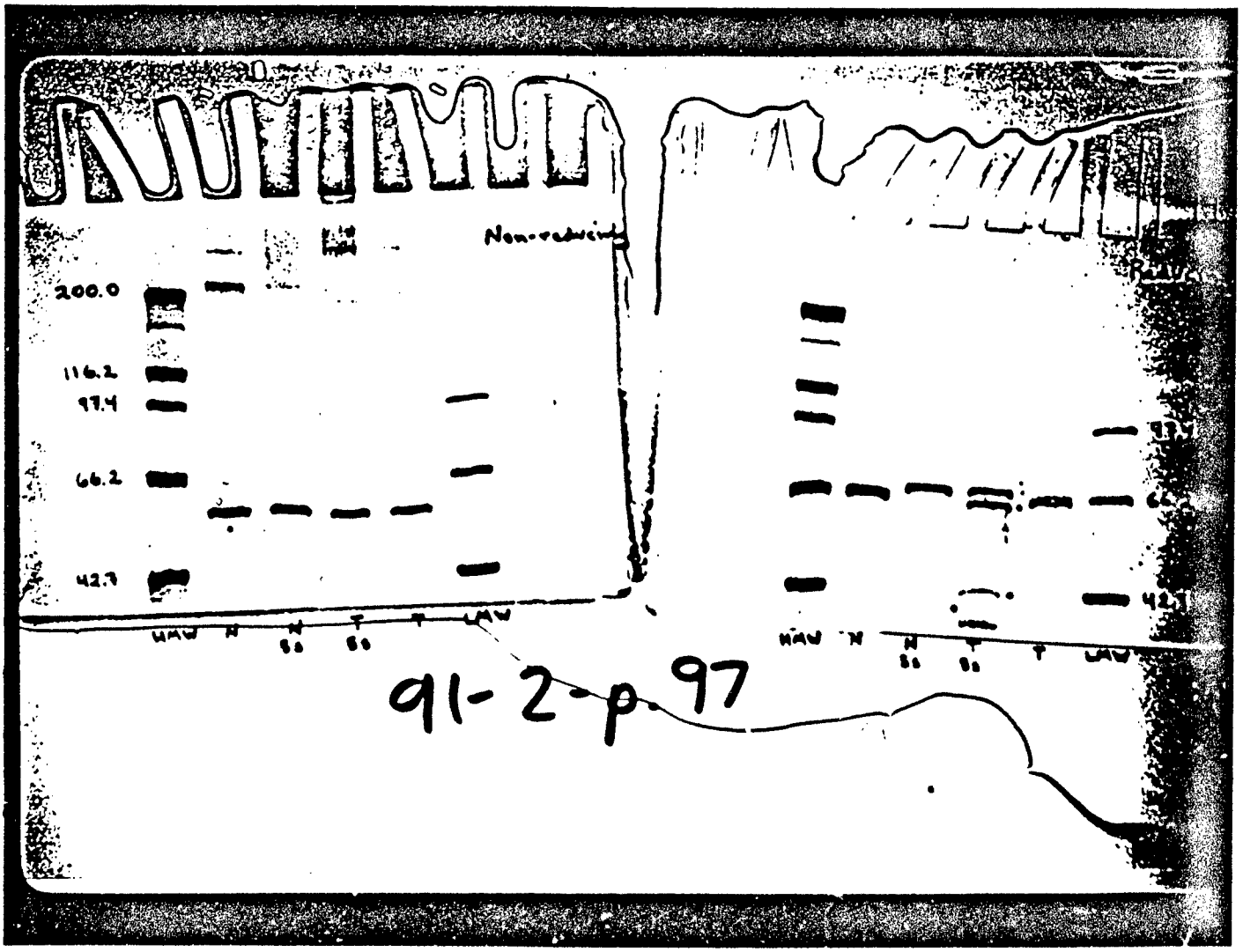

Figure 8

SDS PAGE [illegible] (N) and heated (T) [illegible]
with [illegible] for [illegible] (N 5, T 5) [illegible]
molecular weight (LMW) and high molecular weight (HMW)
[illegible]
[illegible] 42 [illegible] T [illegible]

Complex Formation Between LPS and Acute Phase Rabbit Serum

From: "Interactions of Bacterial Lipopolysaccharide with Acute-Phase Rabbit Serum and Isolation of Two Forms of Rabbit Serum Amyloid A". The Journal of Immunology, 128 (3):1420-1427

Complex 1.2

Density < 1.20 g/cm^3
Binds up about 80% of the added LPS
Serum capacity 500 to 600 µg LPS/ml acute phase serum
Simple saturation occurs beyond the addition of about 800 µg LPS/ml acute phase serum
Complex forms in normal or acute phase serum
Requires EDTA for formation in vitro
Apparent molecular weight: 3.9×10^7
Largely composed of the apolipoproteins expected for HDL's: apolipoprotein A-I, serum amyloid A, three of the serum apolipoprotein C's

Complex 1.3

Density = 1.3 +/- 0.02 g/cm^3
Binds up about 20% of the added LPS
Serum capacity: 1.5 to 2.0 µg LPS/ml acute phase serum
Complex saturation curve with maximum complex formation at addition of 10 µg LPS/ml acute phase serum
Complex only seen in acute phase serum
Does not require EDTA for formation in vitro
Apparent molecular weight: 9.5×10^6
Largely lipid free. Major bands in reducing gel are at MW 91, 64, 61, 50 kD.

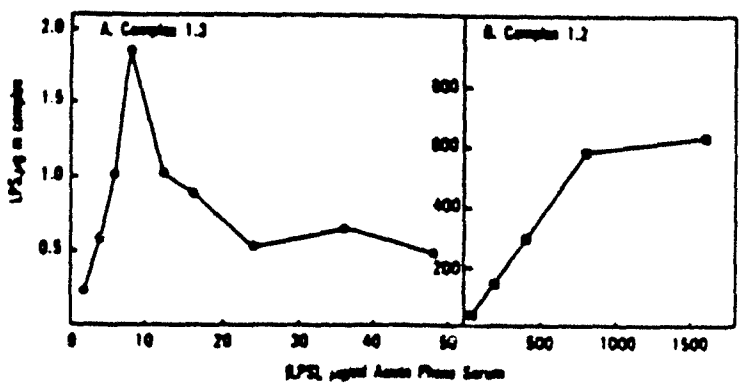

Figure 2. The capacity of acute phase serum to form complexes 1.3 and 1.2. Reaction mixtures of varying amounts of R595 LPS in acute phase serum were centrifuged to equilibrium in CsCl density gradients. The quantity of R595 LPS in each complex was measured by liquid scintillation counting after fractionation of the gradients. *A*, complex 1.3; *B*, complex 1.2.

Figure 9